BARD Final Report US-4828-15

What shapes the crossover landscape in maize and wheat and how can we modify it

Pawlowski, W.P. Cornell University
Levy, A.A. Weizmann Institute of Science

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Three year research project

Abstract

Meiotic recombination is a process in which homologous chromosomes engage in the exchange of DNA segments, creating gametes with new genetic makeup and progeny with new traits. The genetic diversity generated in this way is the main engine of crop improvement in sexually reproducing plants. Understanding regulation of this process, particularly the regulation of the rate and location of recombination events, and devising ways of modifying them, was the major motivation of this project. The project was carried out in maize and wheat, two leading crops, in which any advance in the breeder's toolbox can have a huge impact on food production. Preliminary work done in the USA and Israeli labs had established a strong basis to address these questions. The USA lab pioneered the ability to map sites where recombination is initiated via the induction of double-strand breaks in chromosomal DNA. It has a long experience in cytological analysis of meiosis. The Israeli lab has expertise in high resolution mapping of crossover sites and has done pioneering work on the importance of epigenetic modifications for crossover distribution. It has identified genes that limit the rates of recombination. Our working hypothesis was that an integrative analysis of double-strand breaks, crossovers, and epigenetic data will increase our understanding of how meiotic recombination is regulated and will enhance our ability to manipulate it. The specific objectives of the project were:

(1) To analyze the connection between double-strand breaks, crossover, and epigenetic marks in maize and wheat. Protocols developed for double-strand breaks mapping in maize were applied to wheat. A detailed analysis of existing and new data in maize was conducted to map crossovers at high resolution and search for DNA sequence motifs underlying crossover hotspots. Epigenetic modifications along maize chromosomes were analyzed as well. Finally, a computational analysis tested various hypotheses on the importance of chromatin structure and specific epigenetic modifications in determining the locations of double-strand breaks and crossovers along chromosomes.

(2) Transient knockdowns of meiotic genes that suppress homologous recombination were carried out in wheat using Virus-Induced Gene Silencing. The target genes were orthologs of *FANCM*, *DDM1*, *MET1*, *RECQ4*, and *XRCC2*.

Summary Sheet

Publication Summary

PubType	IS only	Joint	US only
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Training Summary

Trainee Type	Last Name	First Name	Institution	Country
Ph.D. Student	Zhou	Adele	Cornell University	USA
Ph.D. Student	Olson	Mischa	Cornell University	USA
Postdoctoral Fellow	Raz	Amir	Weizmann Institute	Israel
Postdoctoral Fellow	Shilo	Shay	Weizmann Institute	Israel
Postdoctoral Fellow	Wang	Minghui	Cornell University	USA

Contribution of Collaboration

1. The Israeli and US labs shared their datasets on CO location in maize.

2. Dr. Amir Raz, postdoctoral research associate in the Israeli lab, was hosted by the US lab for two weeks in June 2017. During his stay at Cornell, Dr. Raz learned the chromatin immunoprecipitation (ChIP) method to map meiotic DSBs as well as three-dimensional immunolocalization techniques to localize DSBs under the microscope. He also learned how to conduct DSB mapping experiments.

3. The Israeli lab provided the US lab with know-how on how to establish a VIGS transformation system in maize.

3. Data generated by the US and Israeli labs were combined in a joined publication that appeared in PNAS (He *et al.*, 2017).

4. A joint publication on the machine learning analysis of recombination sites, which includes data from both labs, is in preparation and will be submitted in early 2019.

BARD US-4828-15 FINAL SCIENTIFIC REPORT

ACHIEVEMENTS

Objective 1: To analyze the connection between DNA double-strand breaks (DSBs), crossovers (COs), and epigenetic marks in maize and wheat.

Summary

The goal of Objective 1 was to examine the relationship between the distribution of recombination sites, DSBs and COs, and chromatin features. We focused our analysis on maize, as this is the species where a lot more data are available on genome-wide chromatin characteristics. To do the analyses, we set up an extensive database of DSB and CO sites, as well as chromatin characteristics. To add up to the existing data, we performed a genome-wide assay of chromatin sites harboring a heterochromatin mark H3K9me2 in meiotic anthers using chromatin immunoprecipitation. From conducting immunolocalization analyses to follow the dynamics of recombination sites in meiosis, we learned that most recombination events, both DSBs and COs, are formed at sites that are neither heavily euchromatic or heterochromatic. DNA methylation has a strong effect on recombination in maize, affecting COs but not DSBs, which suggests that it contributes the decision on which DSBs are repaired as COs. Using machine learning, we found that the known chromatin characteristics are able to explain well the DSB and CO distribution patterns. These data will be useful for informing efforts to alter chromatin characteristics in order to regulate recombination landscape.

Results

Establishing recombination and chromatin modification datasets

To examine the connections between meiotic DSBs as well as COs and epigenetic marks, we first set up a database containing datasets on maize DSBs and COs. The Levy lab compiled data from public sources to generate a dataset of CO sites in maize that can be mapped with high resolution (<2Kb). This dataset was complemented with data from a high-resolution CO and DSB mapping experiments previously generated in the Pawlowski lab (He *et al.*, 2017; Kianian *et al.*, 2018). The chromatin datasets used for the analyses were also derived from a variety of sources. Data on the distribution of a euchromatin mark H3K4me3 and DNA methylation in meiotic cells were previously generated in the Pawlowski lab (He *et al.*, 2017). Within the scope of the BARD project, we generated a genome-wide map of a heterochromatin mark H3K9me2 in meiotic anthers using chromatin immunoprecipitation followed by Illumina sequencing (ChIP-seq).

Examining DNA sequence and chromatin modification characteristics of recombination sites

The Israeli and US labs collaborated to mine the data on meiotic DSB and CO landscapes in maize. One of the main the outcomes of these analyses was identification of a DNA sequence motif associated with the presence of COs (Figure 1). This motif is similar to the DSB sequence motif identified previously by the Pawlowski lab, as well as the Arabidopsis CO motif found by the Levy lab.

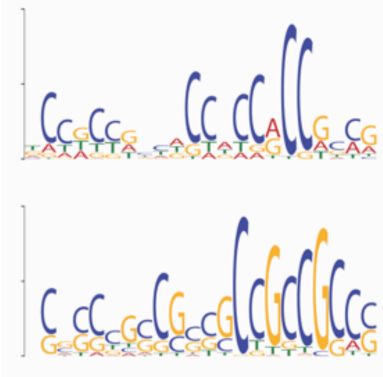

Figure 1. Comparison of CO motifs discovered in maize (top) and Arabidopsis (bottom).

Using recombination site data as well as data on cytosine methylation, the Levi lab showed that both DSBs and COs occur at hypomethylated sites of the maize genome (Figure 2).

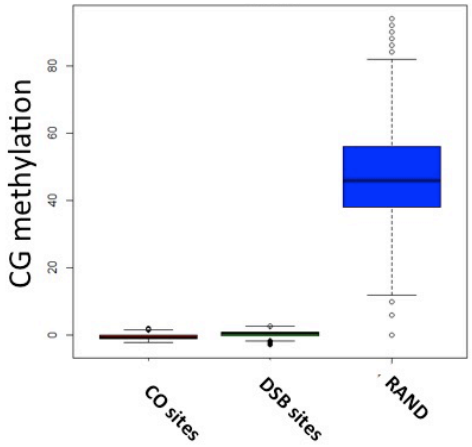

Figure 2. Box plot of methylation in CO sites (n=104) and DSB sites (n=3001), compared to a random set of genome sites (blue box, n=104). All sites were defined with a resolution of 2kb. Low levels of methylation are observed for both CO and DSB sites with a great difference from what is seen in the random dataset (*P*-value =0; U-test).

The US lab furthered the investigation of the effect of heterochromatin on the recombination landscape by comparing genome-wide ChIP-seq profiles of a euchromatin mark H3K4me3 and a heterochromatin mark H3K9me2 to the maps of meiotic DSBs and COs. We also conducted analyses of recombination dynamics using the anti-H3K4me3 and anti- H3K9me2 antibodies as well as an antibody against the RAD51 protein, which marks the sites of meiotic DSBs. The goal of these analyses was to determine whether the type of chromatin that becomes the site of DSB formation changes during the period when DSBs are made in early meiotic prophase. We found that close to 85% of meiotic DSBs in the B73 inbred were neither associated with H3K4me3 nor with H3K9me2. Moreover, during the progression of meiotic prophase the fractions of DSBs associated with H3K4me3 and H3K9me2 remained consistently low. However, later recombination intermediates marked with the HEI10 protein and COs themselves were more frequently associated with H3K4me3 sites than DSBs were. In the case of COs the overlap was almost

50%. These data suggest that chromatin environment does not exert a strong effect on DSB formation, and particularly on the timing when DSBs are made. However, later processes leading to CO formation prefer DSBs formed in H3K4me3-marked euchromatic regions.

Examining the effect on recombination of DNA methylation reduction using the *zmet2* mutant

To further examine the impact of DNA methylation on recombination, the US lab examined the effect of mutating *Zmet2*, one of two chromomethyl transferases in maize. The *zmet2* mutant shows a ca 20% decrease in DNA methylation at CHG sites (Li *et al.*, 2014). Using anti-RAD51 antibody, we found that the number of meiotic DSBs in the mutant was similar to that in the wild-type. To examine CO numbers, we examine chromosomal foci of the HEI10 proteins, which marks CO intermediates (Chelysheva *et al.*, 2012; Wang *et al.*, 2012). Using an anti-HEI10 antibody, we found that the number of HEI10-marked CO intermediates in the *zmet2* mutant was roughly 50% higher than this in wild-type meiocytes.

Using machine learning to dissect recombination landscape

We used our databases of DSB and CO sites to dissect recombination landscapes with machine learning. The goal of these analyses was to determine whether the current collection of chromatin and DNA sequence features implicated in affecting recombination site locations in our studies and studies from other groups (Table 1) is sufficient to understand the recombination landscape, or whether there still remain many features to be discovered.

Table 1. Genome and chromatin features implicated to be associated with recombination sites.

Genome features	Chromatin features
✓ SNPs density (for CO only)	✓ H3K4me3
✓ AT/GC content	✓ H3K9me2
✓ Dinucleotide repeat frequency	✓ H3K27me3
✓ DNA sequence motif presence	✓ H3K5ac
✓ Exon overlap	✓ H3K56ac
✓ Single-exon gene overlap	✓ DNA methylation
✓ Transcript overlap	✓ Micrococcal nuclease (MNase)
✓ Overlap with genes with meiosis-specific expression	✓ Transposase Accessible Chromatin (ATAC)
✓ Distance to transcription start sites (TSS)	
✓ Distance to transcript termination sites (TTS)	
✓ Distance to telomere	
✓ Distance to centromere	
✓ DNA structure (minor groove width, roll, propeller twist, and helix twist)	

Using Principle Component (PC) analysis, we found that the overall characteristics of recombination sites are distinct from those of the rest of the genome (Figure 3).

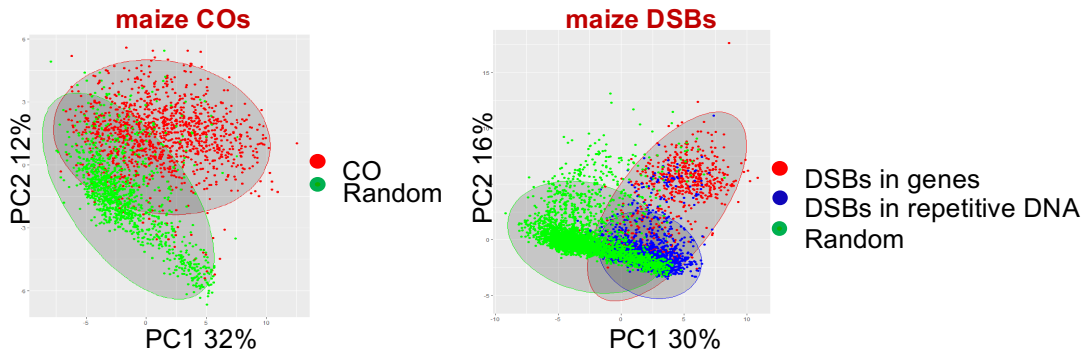

Figure 3. Principle Component analysis of characteristics of meiosis recombination sites.

Overall, we found that the known characteristics of recombination sites were quite sufficient to explain their location in the genome. We examined several machine learning algorithms and found they all performed quite well, being able to detect ~90% of the empirically found DSB and CO sites (Figure 4).

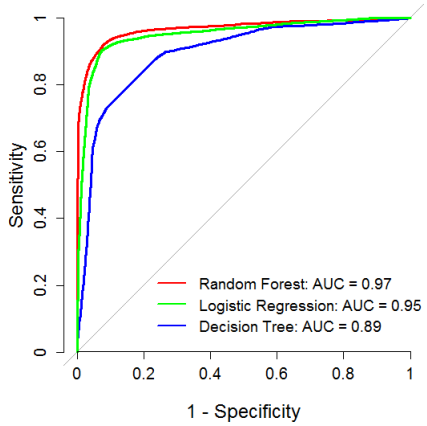

Figure 4. A comparison of the ability of several machine learning algorithms to predict CO site locations in maize.

We found that certain characteristics exhibited more influence on recombination site locations than others (Figure 5). Interestingly, the most important features affecting location were different for DSB sites than for CO sites.

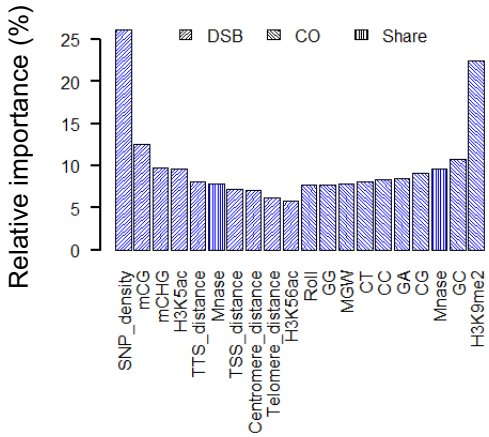

Figure 5. A comparison of genome and chromatin features affecting the location of DSB and CO sites in maize.

We also discovered that the algorithm "learned" from our B73 x Mo17 CO mapping population was able to efficiently predict COs in other maize populations, including NAM, which is a collection of recombinant inbred populations generated by crossing 25 diverse inbred parents to B73 (Figure 6). These results indicate that the relationships between genome/chromatin characteristics and CO sites are genotype-independent.

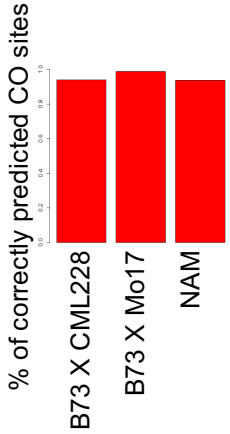

Figure 6. A comparison of the ability of the B73 x Mo17 data to predict CO sites in other maize populations.

Objective 2: To develop transient knockdowns of meiotic genes acting to limit homologous recombination using Virus-Induced Gene Silencing (VIGS).

Summary

Our goal was to silence genes that are putative suppressors of recombination, during meiosis, when recombination between homologues occurs and to measure their effect on meiotic recombination rates in wheat. The genes that were silenced, using VIGS, were: *DDM1, FANCM, MET1,* and *RECQ4* and *XRCC2*. Our main findings are that progenies of F_1 plants treated with MET1- VIGS as well as DDM1-VIGS showed increase in recombination of 76% and 94% respectively at the short arm in sub-telomeric region but not in the pericentric region. XRCC2-VIGS progenies showed an increase of 82% in the long arm sub-telomeric region and, interestingly, a 57%

increase in the pericentric region. Silencing of the other genes had no effect. This provides a simple, non-transgenic tool to enhance meiotic recombination rates in certain regions of the genome, with no need for mutants and in principle in any hybrid done in a breeding program.

Results

Setting up the VIGS system for wheat meiosis

We checked the expression of these genes, in anthers, at the developmental stages of meiosis. Meiosis in wheat occurs during the early booting stage, therefore we sampled anthers from different booting stages as well as heading and mature spikes. We checked the expression levels of two meiotic markers- *SPO11* and *RAD51*, as well as *FANCM* and *XRCC2*, two of our target genes. As shown in Figure 7, the level of expression of meiotic genes in anthers starts to increase already at boot1 stage (in comparison with non-meiotic mature anthers) reaching the highest levels between boot3 and heading, and going down after emergence of the spike (mature stage). To be on the safe side we decided to sample anthers between boot2 and boot 3, considering also the fact that zygotene occurs during boot2 stage as proven by chromosome staining.

Figure 7. Meiotic genes expression at different developmental stages. Bottom – five developmental stages of the spike. Upper – relative expression levels of the meiotic genes *SPO11* (blue), *FANCM* (purple), *RAD51* (green), *XRCC2* (orange).

Virus-induced Gene Silencing of recombination suppressors

We infected tillers with the engineered BSMV (Figure 8A) two to three weeks before anthesis, usually on the third or fourth leaf, using both needle-less infiltration and the rubbing method (Lee *et al.*, 2015). Anthers from three different spikelets were sampled at booting stage2 and tested for the expression levels of each gene by qPCR to assess the silencing effect. As shown in Figure 8A VIGS worked well on *MET1* and *XRCC2* genes, reducing their expression level by 65% and 39% respectively ($P<0.05$) compare to WT plants (Figure 8B). Empty vector showed some non-significant reduction in gene expression, possibly due to the stress effect of the virus infection. Unfortunately, reduction in expression of the *FANCM* and *RECQ4* genes were not significant. Since in preliminary experiment *FANCM* showed good silence effect, we performed another experiment for silencing *FANCM* as well as *DDM1*. In this experiment, we also added a combined treatment, infecting plant with both VIGS-FANCM and VIGS-DDM1 viruses. This time *FANCM* expression was reduced by 45% while *DDM1* expression was reduced by 24% ($P<0.05$). Surprisingly, the

combined treatment had an even stronger effect, reducing expression levels of both genes by 85% and 48% respectively (Figure 8C).

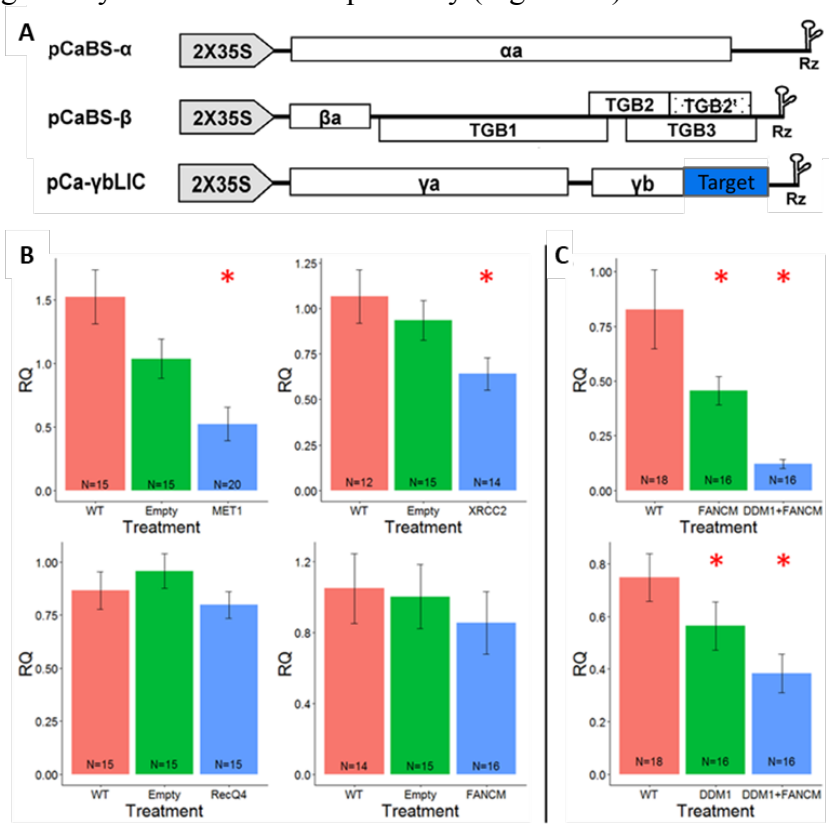

Figure 8. Relative expression levels after infection with different VIGS treatments. A – VIGS constructs. Each of the three BSMV sub-genomes was cloned into the pCass4-Rz binary vector under the 35S promoter. Target – GOI sequence. Adapted from (Yuan *et al.*, 2011). B – First experiment. C – Second experiment. WT – un-infected plants. Empty – infection with empty virus. Asterisks designate significant differences from WT (P <0.05).

Number of seeds

In order to check whether the silencing treatment had a lethal effect on the gametes or the developing seeds, we counted the number of seeds in the treated spikes (Figure 9). In the first experiment, only RECQ4-VIGS treatment showed a significant effect on fertility, reducing the seeds number down to two seeds per spike, compared to 19 seeds in the WT. The other treatments showed only mild, non-significant reduction, probably due to the viral infection stress. In the second experiment, all treatments showed significant reduction in seeds number, reaching 5-7 seeds per spike compared to 19 seeds in the WT, implying a lethal effect of silencing these genes.

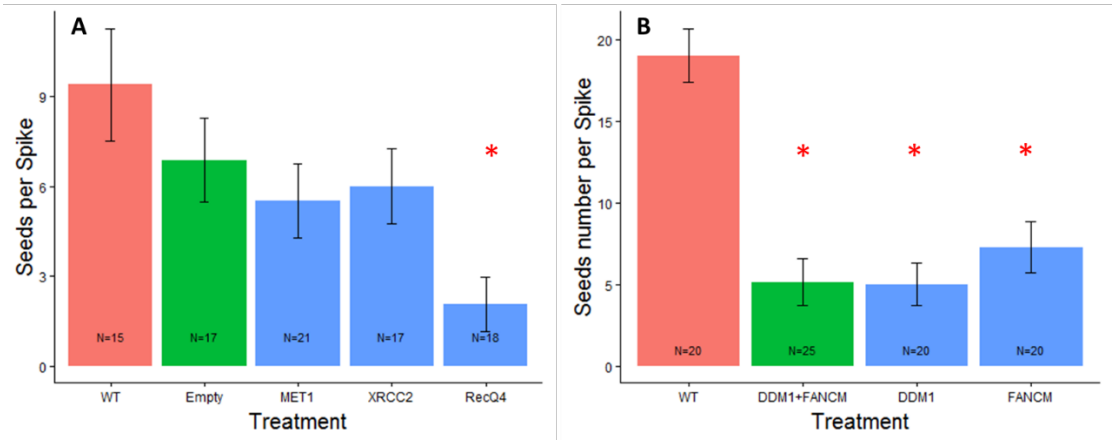

Figure 9. Mean seeds number in VIGS treated spikes. A – First experiment. B – Second experiment. Asterisks designate significant differences from WT (P<0.05).

Analysis of meiotic recombination using INDEL markers.

To analyze recombination rate in the F_2 progenies we developed a series of Indel markers, that are easy to screen for, through a whole genome comparison of the 'Zavitan' and 'Svevo' genomes. We focused on chromosome 1A, where we choose ten InDel markers along the chromosome (Figure 10A). All markers have a 100-200 bp larger 'Zavitan' product, so that a simple gel-electrophoresis was sufficient for genotyping. We selected three pairs of markers with genetic distance of 9 to 22 cM: one for each sub-telomeric region and another one spanning the pericentric region. We used these three intervals to look for increase in recombination rate (Figure 10B). Progenies of F_1 plants treated with MET1-VIGS as well as DDM1-VIGS showed increase in recombination of 76% and 94% respectively at the short arm in sub-telomeric region but not in the other intervals. XRCC2-VIGS progenies showed an increase of 82% in the long arm sub-telomeric region and, interestingly, a 57% increase in the pericentric region. In the first experiment, treatment with FANCM-VIGS showed no significant changes in recombination, which correlate with the non-significant silencing effect. Similarly, in the second experiment, FANCM-VIGS failed to show any changes in recombination rates, even though the silencing effect was now significant. However, in the combined DDM1+FANCM-VIGS treatment a mild non-significant increase of 27% was found at the long arm sub-telomeric region. In order to check the total number of recombination events in chromosome 1A, we used 10 InDel markers along the chromosome to identify all events in each progeny. We found no overall increase of recombination events in any of the treatments but rather redistribution of CO sites (Figure 11).

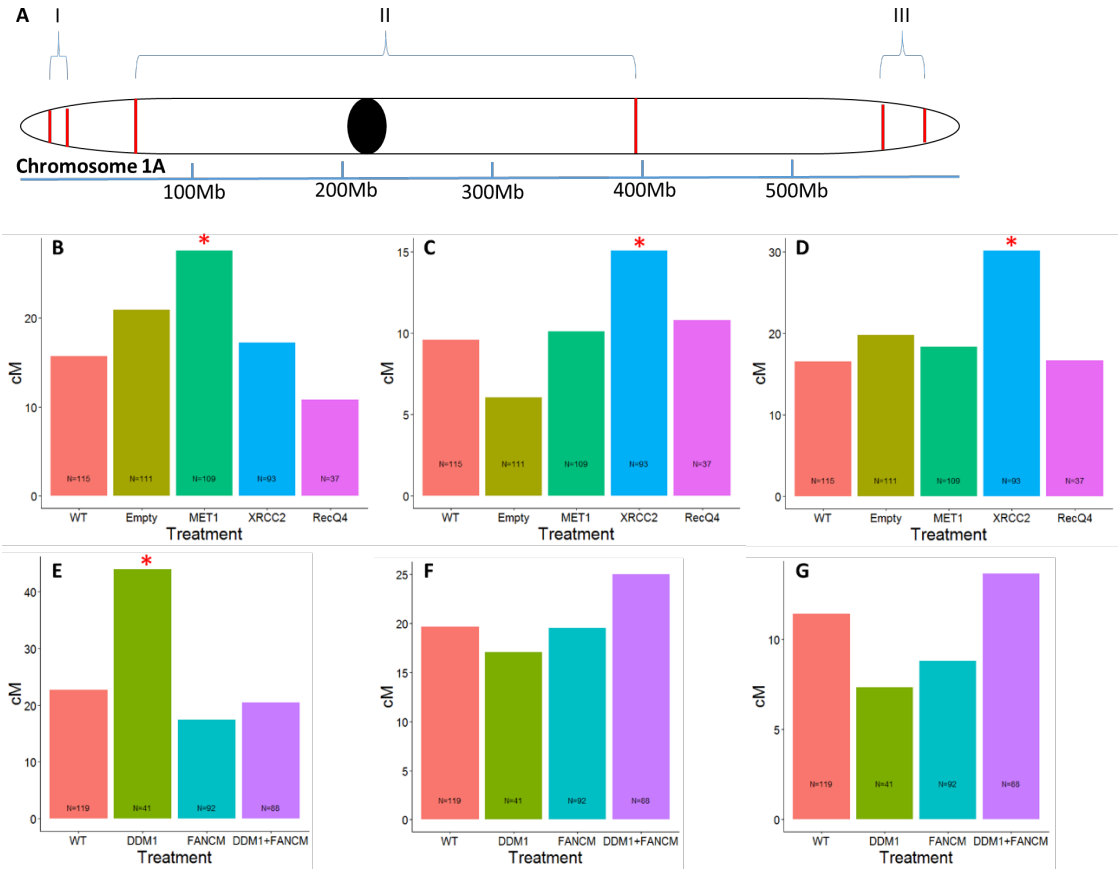

Figure 10. Genetic distance between three pairs of markers on chromosome 1A, after VIGS treatment. A – Schematic map of the three intervals measured for changes in genetic distance. I - short arm sub-telomeric region, II - peri-centric region, III - long arm sub-telomeric region. B, E – changes at the short arm sub-telomeric region. C, F – changes at the peri-centric region. D, G - changes at the long arm sub-telomeric region. Asterisk designate significant differences from WT (P <0.05).

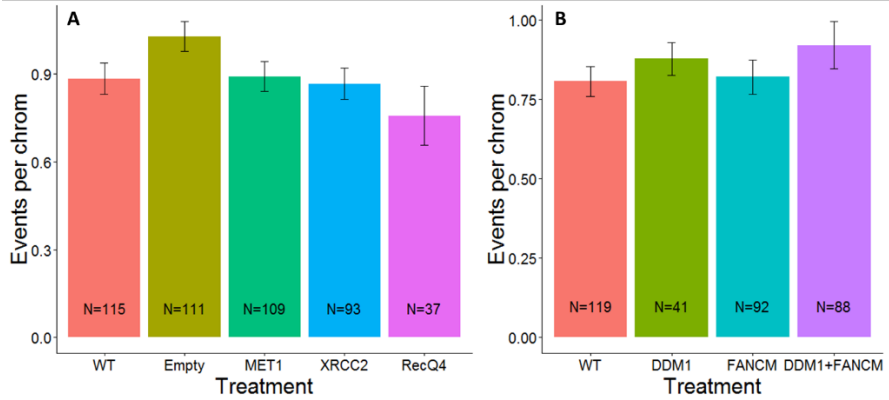

Figure 11: mean number of recombination events along chromosome 1A after VIGS treatment. Asterisks designate significant differences from WT (P <0.05).

Allelic distortion

Allele distortion represent the changes in the different genotypes distribution from the expected Mendelian distribution of 1:2:1 between both homozygote and heterozygote genotypes or from the 1:1 distribution of the parental alleles in the population. We checked the allele distortion in the different VIGS populations in comparison to the WT population. We found allele distortion in the MET1-VIGS and RECQ4-VIGS

populations where a significant increase of "Zavitan" allele and decrease of 'Svevo' allele was found along most of the chromosome except from the sub-telomeric regions (Figure 12). In the second experiment, a similar but milder distortion was found in both DDM1-VIGS and DDM1+FANCM-VIGS populations. However, this behavior was also found in the WT population, implying that a simple selection pressure rather than VIGS silencing effect is responsible for the distortion in this experiment.

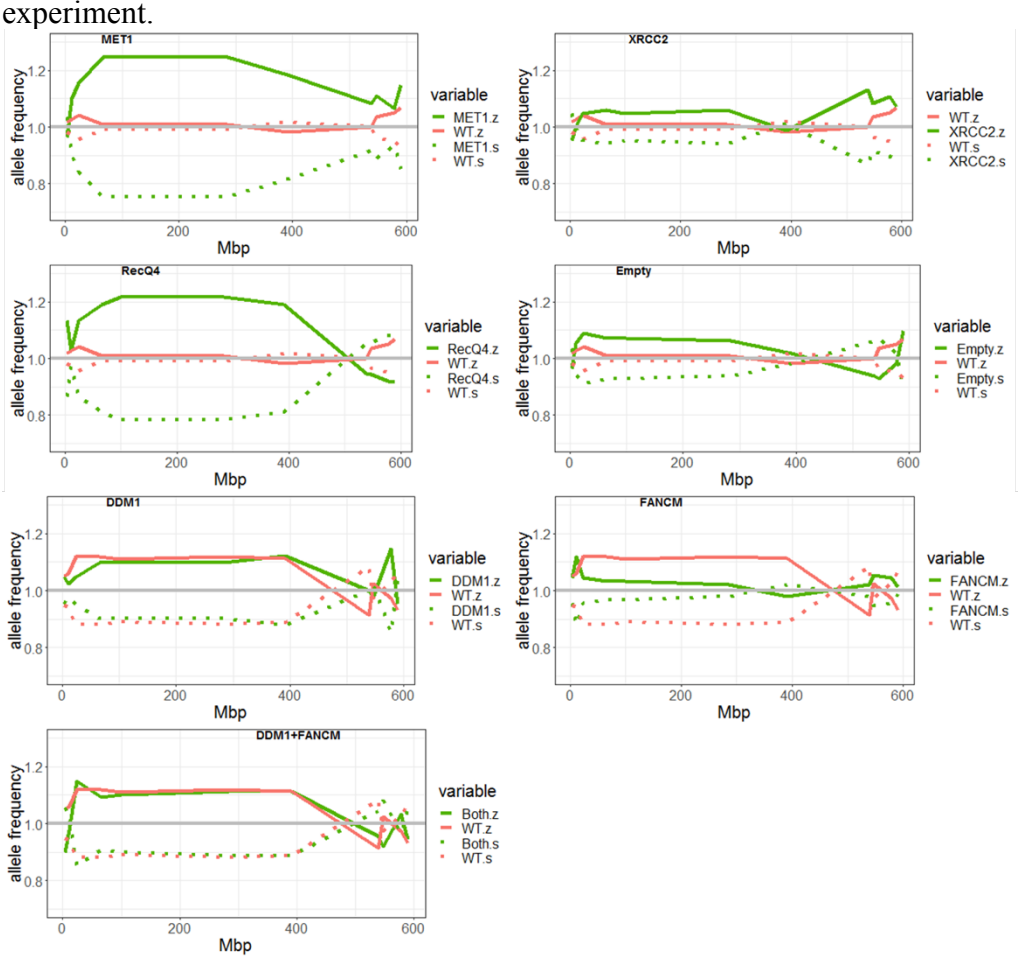

Figure 12. **A**llele distortion of 'Zavitan' and 'Svevo' allele along chromosome 1A after VIGS treatment. Lines – 'Zavitan' allele. Dots – 'Svevo' allele. Red- WT, Green – VIGS treatment. Asterisks designate significant differences from WT (P<0.05).

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Publications for Project US-4828-15

Status	Type	Authors	Title	Journal	Vol:pg Year	Coun
Published	Reviewed	*He Y., Wang M., Dukowic-Schulze S., Zhou A., Tiang C.-L., Shilo S., Sidhu G.K., Eichten S., Bradbury P., Springer N.M., Buckler E., LevyA.A., Qi S., Pillardy J., Kianian P.M.A., Kianian S., Chen C., and Pawlowski W.P.*	Genomic features shaping the landscape of meiotic double strand break hotspots in maize.	***PNAS***	114 : 12231-12236 2017	Joint